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ACTIVATED FORMS OF NOTCH AND METHODS BASED THEREON

This application is a continuation-in-part application of co-pending United States Application Serial No. 08/899,232 filed July 23, 1997, which is incorporated by reference herein in its entirety.

This invention was made with government support under grant number NS 26084 awarded by the National Institutes of Health. The government has certain rights in the invention.

1. FIELD OF THE INVENTION

The present invention is directed to methods for detecting or measuring Notch activation by observing or measuring the appearance of Notch on the cell surface or by observing or measuring Notch cleavage products that are indicative of Notch activation. The present invention is also directed to methods for detecting a molecule that modulates Notch activation by observing or measuring a change in the amount of Notch expressed on the cell surface or a change in the amount or pattern of Notch cleavage products. The present invention is also directed to a substantially purified activated heterodimeric form of Notch and pharmaceutical compositions and kits thereof.

2. BACKGROUND OF THE INVENTION

Genetic and molecular studies have led to the identification of a group of genes which define distinct elements of the Notch signaling pathway. While the identification of these various elements has come exclusively from *Drosophila* using genetic tools as the initial guide, subsequent analyses have lead to the identification of homologous proteins in vertebrate species including humans. Figure 1 depicts the molecular relationships between the known Notch pathway elements as well as their subcellular localization (Artavanis-Tsakonas et al., 1995, Science 268:225-232).

The *Drosophila Notch* gene encodes an ~300 kD transmembrane protein that acts as a receptor in a cell-cell signaling mechanism controlling cell fate decisions throughout development (reviewed, *e.g.*, in Artavanis-Tsakonas et al., 1995, Science 268:225-232). Closely related homologs of *Drosophila Notch* have been isolated from a number of vertebrate species, including humans, with multiple paralogs representing the single *Drosophila* gene in vertebrate genomes. The isolation of cDNA clones encoding the C-terminus of a human Notch paralog, originally termed hN, has been reported (Stifani et al., 1992, Nature Genetics 2:119-127). The encoded protein is designated human Notch2 because of its close relationship to the Notch2 proteins found in other species (Weinmaster et al., 1992, Development 116:931-941). The hallmark Notch2 structures are common to all the Notch-related proteins, including, in the extracellular domain, a stretch of 34 to 36 tandem Epidermal Growth Factor-like (EGF) repeats and three Lin-12/Notch repeats (LN repeats), and, in the intracellular domain, 6 Ankyrin repeats and a PEST-containing region. Like *Drosophila Notch* and the related *C. elegans* genes *lin-12* and *glp-1* (Sternberg, 1993, Current Biology 3:763-765; Greenwald, 1994, Current Opinion in Genetics and Development 4:556-562), the vertebrate Notch homologs play a role in a variety of developmental processes by controlling cell fate decisions (reviewed, *e.g.*, in Blaumueller and Artavanis-Tsakonas, 1997, Persp. on Dev. Neurobiol. 4:325-343). (For further human Notch sequences, see International Publication WO 92/19734.)

The extracellular domain of Notch carries 36 Epidermal Growth Factor-like (EGF) repeats, two of which (repeats 11 and 12) have been implicated in interactions with the Notch ligands Serrate and Delta. Delta and Serrate are membrane bound ligands with EGF homologous extracellular domains, which interact physically with Notch on adjacent cells to trigger signaling.

Functional analyses involving the expression of truncated forms of the Notch receptor have indicated that

receptor activation depends on the six cdc10/ankyrin repeats in the intracellular domain. Deltex and Suppressor of Hairless, whose over-expression results in an apparent activation of the pathway, associate with those repeats.

Deltex is a cytoplasmic protein which contains a ring zinc finger. Suppressor of Hairless on the other hand, is the *Drosophila* homologue of CBF1, a mammalian DNA binding protein involved in the Epstein-Barr virus-induced immortalization of B cells. It has been demonstrated that, at least in cultured cells, Suppressor of Hairless associates with the cdc10/ankyrin repeats in the cytoplasm and translocates into the nucleus upon the interaction of the Notch receptor with its ligand Delta on adjacent cells (Fortini and Artavanis, 1994, Cell 79:273-282). The association of Hairless, a novel nuclear protein, with Suppressor of Hairless has been documented using the yeast two hybrid system; therefore, it is believed that the involvement of Suppressor of Hairless in transcription is modulated by Hairless (Brou et al., 1994, Genes Dev. 8:2491; Knust et al. 1992, Genetics 129:803).

Finally, it is known that Notch signaling results in the activation of at least certain basic helix-loop-helix (bHLH) genes within the Enhancer of Split complex (Delidakis et al., 1991, Genetics 129:803). Mastermind encodes a novel ubiquitous nuclear protein whose relationship to Notch signaling remains unclear but is involved in the Notch pathway as shown by genetic analysis (Smoller et al., 1990, Genes Dev. 4:1688).

The generality of the Notch pathway manifests itself at different levels. At the genetic level, many mutations exist which affect the development of a very broad spectrum of cell types in *Drosophila*. Knockout mutations in mice are embryonic lethals consistent with a fundamental role for Notch function (Swiatek et al., 1994, Genes Dev. 8:707). Mutations in the Notch pathway in the hematopoietic system in humans are associated with lymphoblastic leukemia (Ellison et al., 1991, Cell 66:649-661). Finally the expression of

mutant forms of Notch in developing *Xenopus* embryos interferes profoundly with normal development (Coffman et al., 1993, Cell 73:659). Increased level of Notch expression is found in some malignant tissue in humans (International Publication WO 94/07474).

The expression patterns of Notch in the *Drosophila* embryo are complex and dynamic. The Notch protein is broadly expressed in the early embryo, and subsequently becomes restricted to uncommitted or proliferative groups of cells as development proceeds. In the adult, expression persists in the regenerating tissues of the ovaries and testes (reviewed in Fortini et al., 1993, Cell 75:1245-1247; Jan et al., 1993, Proc. Natl. Acad. Sci. USA 90:8305-8307; Sternberg, 1993, Curr. Biol. 3:763-765; Greenwald, 1994, Curr. Opin. Genet. Dev. 4:556-562; Artavanis-Tsakonas et al., 1995, Science 268:225-232). Studies of the expression of Notch1, one of three known vertebrate homologs of Notch, in zebrafish and *Xenopus*, have shown that the general patterns are similar; with Notch expression associated in general with non-terminally differentiated, proliferative cell populations. Tissues with high expression levels include the developing brain, eye and neural tube (Coffman et al., 1990, Science 249:1438-1441; Bierkamp et al., 1993, Mech. Dev. 43:87-100). While studies in mammals have shown the expression of the corresponding Notch homologs to begin later in development, the proteins are expressed in dynamic patterns in tissues undergoing cell fate determination or rapid proliferation (Weinmaster et al., 1991, Development 113:199-205; Reaume et al., 1992, Dev. Biol. 154:377-387; Stifani et al., 1992, Nature Genet. 2:119-127; Weinmaster et al., 1992, Development 116:931-941; Kopan et al., 1993, J. Cell Biol. 121:631-641; Lardelli et al., 1993, Exp. Cell Res. 204:364-372; Lardelli et al., 1994, Mech. Dev. 46:123-136; Henrique et al., 1995, Nature 375:787-790; Horvitz et al., 1991, Nature 351:535-541; Franco del Amo et al., 1992, Development 115:737-744). Among the tissues in which mammalian Notch homologs are first expressed are the pre-somitic mesoderm and the developing

neuroepithelium of the embryo. In the pre-somitic mesoderm, expression of Notch1 is seen in all of the migrated mesoderm, and a particularly dense band is seen at the anterior edge of pre-somitic mesoderm. This expression has been shown to decrease once the somites have formed, indicating a role for Notch in the differentiation of somatic precursor cells (Reaume et al., 1992, Dev. Biol. 154:377-387; Horvitz et al., 1991, Nature 351:535-541). Similar expression patterns are seen for mouse Delta (Simske et al., 1995, Nature 375:142-145).

Within the developing mammalian nervous system, expression patterns of Notch homologue have been shown to be prominent in particular regions of the ventricular zone of the spinal cord, as well as in components of the peripheral nervous system, in an overlapping but non-identical pattern. Notch expression in the nervous system appears to be limited to regions of cellular proliferation, and is absent from nearby populations of recently differentiated cells (Weinmaster et al., 1991, Development 113:199-205; Reaume et al., 1992, Dev. Biol. 154:377-387; Weinmaster et al., 1992, Development 116:931-941; Kopan et al., 1993, J. Cell Biol. 121:631-641; Lardelli et al., 1993, Exp. Cell Res. 204:364-372; Lardelli et al., 1994, Mech. Dev. 46:123-136; Henrique et al., 1995, Nature 375:787-790; Horvitz et al., 1991, Nature 351:535-541). A rat Notch ligand is also expressed within the developing spinal cord, in distinct bands of the ventricular zone that overlap with the expression domains of the Notch genes. The spatio-temporal expression pattern of this ligand correlates well with the patterns of cells committing to spinal cord neuronal fates, which demonstrates the usefulness of Notch as a marker of populations of cells for neuronal fates (Henrique et al., 1995, Nature 375:787-790). This has also been suggested for vertebrate Delta homologs, whose expression domains also overlap with those of Notch1 (Larsson et al., 1994, Genomics 24:253-258; Fortini et al., 1993, Nature 365:555-557; Simske et al., 1995, Nature 375:142-145). In the cases of the

Xenopus and chicken homologs, Delta is actually expressed only in scattered cells within the Notch1 expression domain, as would be expected from the lateral specification model, and these patterns "foreshadow" future patterns of neuronal differentiation (Larsson et al., 1994, Genomics 24:253-258; Fortini et al., 1993, Nature 365:555-557).

Other vertebrate studies of particular interest have focused on the expression of Notch homologs in developing sensory structures, including the retina, hair follicles and tooth buds. In the case of the *Xenopus* retina, Notch1 is expressed in the undifferentiated cells of the central marginal zone and central retina (Coffman et al., 1990, Science 249:1439-1441; Mango et al., 1991, Nature 352:811-815). Studies in the rat have also demonstrated an association of Notch1 with differentiating cells in the developing retina have been interpreted to suggest that Notch1 plays a role in successive cell fate choices in this tissue (Lyman et al., 1993, Proc. Natl. Acad. Sci. USA 90:10395-10399).

A detailed analysis of mouse Notch1 expression in the regenerating matrix cells of hair follicles was undertaken to examine the potential participation of Notch proteins in epithelial/mesenchymal inductive interactions (Franco del Amo et al., 1992, Development 115:737-744). Such a role had originally been suggested for Notch1 based on its expression in rat whiskers and tooth buds (Weinmaster et al., 1991, Development 113:199-205). Notch1 expression was instead found to be limited to subsets of non-mitotic, differentiating cells that are not subject to epithelial/mesenchymal interactions, a finding that is consistent with Notch expression elsewhere.

Expression studies of Notch proteins in human tissue and cell lines have also been reported. The aberrant expression of a truncated Notch1 RNA in human T-cell leukemia results from a translocation with a breakpoint in Notch1 (Ellisen et al., 1991, Cell 66:649-661). A study of human Notch1 expression during hematopoiesis has suggested a role

for Notch1 in the early differentiation of T-cell precursors (Mango et al., 1994, Development 120:2305-2315). Additional studies of human Notch1 and Notch2 expression have been performed on adult tissue sections including both normal and neoplastic cervical and colon tissue. Notch1 and Notch2 appear to be expressed in overlapping patterns in differentiating populations of cells within squamous epithelia of normal tissues that have been examined and are clearly not expressed in normal columnar epithelia, except in some of the precursor cells. Both proteins are expressed in neoplasias, in cases ranging from relatively benign squamous metaplasias to cancerous invasive adenocarcinomas in which columnar epithelia are replaced by these tumors (Mello et al., 1994, Cell 77:95-106).

Insight into the developmental role and the general nature of Notch signaling has emerged from studies with truncated, constitutively activated forms of Notch in several species. These recombinantly engineered Notch forms, which lack extracellular ligand-binding domains, resemble the naturally occurring oncogenic variants of mammalian Notch proteins and are constitutively activated using phenotypic criteria (Greenwald, 1994, Curr. Opin. Genet. Dev. 4:556; Fortini et al., 1993, Nature 365:555-557; Coffman et al., 1993, Cell 73:659-671; Struhl et al., 1993, Cell 69:1073; Rebay et al., 1993, Genes Dev. 7:1949; Kopan et al., 1994, Development 120:2385; Roehl et al., 1993, Nature 364:632).

- Ubiquitous expression of activated Notch in the *Drosophila* embryo suppresses neuroblast segregation without impairing epidermal differentiation (Struhl et al., 1993, Cell 69:331; Rebay et al., 1993, Genes Dev. 7:1949).

- Persistent expression of activated Notch in developing imaginal epithelia likewise results in an overproduction of epidermis at the expense of neural structures (Struhl et al., 1993, Cell 69:331).

- Neuroblast segregation occurs in temporal waves that are delayed but not prevented by transient expression of

activated Notch in the embryo (Struhl et al., 1993, Cell 69:331).

- Transient expression in well-defined cells of the *Drosophila* eye imaginal disc causes the cells to ignore their normal inductive cues and to adopt alternative cell fates (Fortini et al., 1993, Nature 365:555-557).

- Studies utilizing transient expression of activated Notch in either the *Drosophila* embryo or the eye disc indicate that once Notch signaling activity has subsided, cells may recover and differentiate properly or respond to later developmental cues (Fortini et al., 1993, Nature 365:555-557; Struhl et al., 1993, Cell 69:331).

For a general review on the Notch pathway and Notch signaling, see Artavanis-Tsakonas et al., 1995, Science 268:225-232.

Ligands, cytoplasmic effectors and nuclear elements of Notch signaling have been identified in *Drosophila*, and vertebrate counterparts have also been cloned (reviewed in Artavanis-Tsakonas et al., 1995, Science 268:225-232). While protein interactions between the various elements have been documented, the biochemical nature of Notch signaling remains elusive. Expression of truncated forms of Notch reveal that Notch proteins without transmembrane and extracellular domains are translocated to the nucleus both in transgenic flies and in transfected mammalian or *Drosophila* cells (Lieber et al., 1993, Genes and Development 7:1949-1965; Fortini et al., 1993, Nature 365:555-557; Ahmad et al., 1995, Mechanisms of Development 53:78-85; Zagouras et al., 1995, Proc. Natl. Acad. Sci. USA 92:6414-6418). Sequence comparisons between mammalian and *Drosophila* Notch molecules, along with deletion analysis, have found two nuclear localization sequences that reside on either side of the Ankyrin repeats (Stifani et al., 1992, Nature Genetics 2:119-127; Lieber et al., 1993, Genes and Development 7:1949-1965; Kopan et al., 1994, Development 120:2385-2396). These findings prompted the speculation that Notch may be directly participating in nuclear events by means of a proteolytic

cleavage and subsequent translocation of the intracellular fragment into the nucleus. However, conclusive functional evidence for such a hypothesis remained elusive (Artavanis-Tsakonas et al., 1995, Science 268:225-232) until the disclosure of Schroeter et al., 1998, Nature 393:382-386. Schroeter et al. demonstrated that Notch1, upon ligand binding, is cleaved between amino acid G1743 and V1744 releasing the intracellular domain. The released intracellular domain translocates into the nucleus, and through interaction with members of the CSL (CBF-1, Su(H), Lag-1) family of DNA binding proteins, activates transcription.

In a separate study, Logeat et al., 1998, Proc. Natl. Acad. Sci. USA 95:8108-8112 (Logeat et al.), have demonstrated that human Notch1 is constitutively cleaved by the convertase furin at the carboxyl side of the sequence ArgGlnArgArg (amino acids 1651-1654), which sequence is located between the transmembrane domain and the Lin-12/Notch repeats. The cleavage of Notch1 by furin results in the cell surface expression of a heterodimeric functional receptor.

Citation or identification of any reference in Section 2 or any other section of this application shall not be construed as an admission that such reference is available as prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention is directed to methods for detecting or measuring Notch activation by observing or measuring the appearance of Notch on the cell surface or by observing or measuring Notch cleavage products that are indicative of Notch activation. In one aspect of this embodiment of the invention, the method for detecting or measuring Notch activation in a cell comprises detecting or measuring the expression of Notch on the surface of said cell, wherein the presence and amount of Notch on the surface indicates the presence and amount, respectively, of Notch activation. In another aspect, the method comprises

detecting or measuring the expression of one or more Notch cleavage products selected from the group consisting of N^{EC} and N^{TM}. In yet another aspect, the method comprises detecting or measuring one or more fragments of Notch selected from the group consisting of an amino-terminal fragment of full-length Notch terminating between the epidermal growth factor-like repeat domain and the transmembrane domain (in particular, between the Lin-12/Notch repeats and the transmembrane domain) of full-length Notch, and a carboxy-terminal fragment of full-length Notch with its amino terminus situated between the epidermal growth factor-like repeat domain and the transmembrane domain (in particular, between the Lin-12/Notch repeats and the transmembrane domain), or detecting or measuring one or more fragments of Notch selected from the group consisting of Notch fragments having a molecular weight of about 270, 200, 170, 140, 110, 100, 90 and 85 kilodaltons. In yet another aspect, the method comprises detecting or measuring a Notch heterodimer containing a reducing agent-sensitive linkage, in particular, a non-covalent, metal ion-dependent (*e.g.*, calcium ion-dependent) linkage.

The present invention is based, at least in part, on the discovery that Notch in its active form, *i.e.*, the form that mediates signal transduction and that binds Notch ligands such as Delta, is a heterodimer of two Notch cleavage products, an about (±10%) 180 kilodaltons (kDa) subunit (N^{EC}) and an about (±10%) 110 kDa subunit (N^{TM}), which are tethered together through a reducing agent-sensitive linkage, in particular, a non-covalent, metal ion-dependent (*e.g.*, calcium ion-dependent) linkage. Full length Notch is not expressed on the cell surface and is ligand inaccessible. As shown by way of example *infra*, the two subunits arise due to a proteolytic cleavage of the full length Notch molecule in the trans-Golgi at a site in Notch amino-terminal to the transmembrane domain and carboxy-terminal to the EGF repeat region, thus generating an extracellular fragment (N^{EC}) of about 180 kDa and a transmembrane/intracellular fragment (N^{TM})

of about 110 kDa. The detection of full-length Notch and of Notch cleavage products, as well as Notch that is present on the cell surface, can be carried out by methods well known to those of skill in the art, *e.g.*, precipitation or binding to an immobilized binding partner (*e.g.*, on a plate or column), *e.g.*, anti-Notch antibodies or ligands of Notch, such as Delta and Serrate.

The detection or measurement of Notch activation is important in the study and manipulation of differentiation processes, since Notch plays a key role in cell fate (differentiation) determination. Also, disorders of cell fate, in particular hyperproliferative (*e.g.*, cancer) or hypoproliferative disorders, involving aberrant or undesirable levels of active Notch expression can be diagnosed or screened for by detecting such active Notch expression, as described more fully *infra*. Molecules that modulate Notch function are important tools for studying and manipulating differentiation processes, *e.g.*, in expanding cell populations without substantial differentiation (International Publication WO 97/11716), in cancer studies and therapy (International Publication WO 94/07474), and differentiation studies on normal tissue.

In another embodiment, the present invention is also directed to methods for identifying a molecule that modulates Notch activation by detecting or measuring a change in the amount of Notch expressed on the cell surface or a change in the amount or pattern of Notch cleavage products. In one aspect of this embodiment of the invention, the method for identifying a modulator of Notch activation comprises providing a cell with a candidate modulator molecule and detecting or measuring the amount of Notch on the surface of the cell, in which a difference in the presence or amount compared to a cell not contacted with the candidate molecule indicates that the candidate molecule modulates Notch activation. In another aspect, the method for identifying a modulator of Notch activation comprises providing a cell with a candidate modulator molecule and detecting or measuring the

expression by the cell of one or more Notch cleavage products selected from the group consisting of N^{EC} and N^{TM}, in which a difference in the presence or amount of said one or more cleavage products compared to a Notch cell not contacted with the candidate molecule indicates that the molecule modulates Notch activity.

In an alternative aspect, the method for identifying a modulator of Notch activation comprises contacting a candidate modulator molecule with a full length Notch in the presence of a composition comprising cellular proteins, under conditions conducive to cleavage of the full-length Notch by one or more components of the composition and detecting or measuring the amount of Notch cleavage products N^{EC} and N^{TM} that result, in which a difference in the presence or amount of said Notch cleavage products compared to a full-length Notch in presence of said composition not contacted with the candidate molecule indicates that the molecule modulates Notch activity.

The present invention is also directed to a substantially purified active form of Notch which comprises Notch fragments tethered together through a reducing agent-sensitive linkage, particularly, a non-covalent, metal ion-dependent linkage, and pharmaceutical compositions and kits thereof.

4. BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic diagram of the Notch signaling pathway. The Notch receptor can bind to either Delta or Serrate through its extracellular domain. Ligand binding can result in receptor multimerization that is stabilized by interactions between the intracellular ankyrin repeats of Notch and the cytoplasmic protein Deltex. These events can control the nuclear translocation of the DNA-binding protein Suppressor of Hairless and its known association with the Hairless protein. The transcriptional induction of the Enhancer of Split basic helix-loop-helix (bHLH) genes appears to depend on Notch signaling.

Figure 2 is a Notch homolog sequence comparison. The human Notch2 (humN2) (SEQ ID NO:1), human Notch1 (humN1) (SEQ ID NO:2), *Xenopus* Notch/Xotch (XenN) (SEQ ID NO:3), and *Drosophila* Notch (DrosN) (SEQ ID NO:4) protein sequences are aligned, with names indicated to the left and numbering to the right (Wharton et al., 1985, Cell 43:567-581; Coffman et al., 1990, Science 249:1438-1441; Ellisen et al., 1991, Cell 66:649-661; Stifani et al., 1992, Nature Genetics 2:119-127). Major Notch protein motifs are enclosed in boxes. Starting from the N-terminal, the boxed regions indicate: EGF repeats, Lin-12/Notch (LN) repeats, transmembrane domain (TM), Ankyrin repeats, and PEST-containing region. Also indicated are the putative CcN motif components (Stifani et al., 1992, Nature Genetics 2:119-127) nuclear localization signal (NLS, BNTS) and putative CKII and cdc2 phosphorylation sites. The calculated signal cleavage site is indicated with an arrow.

Figures 3A-3E are Western blot analyses of human cell lines, human tissues, *Drosophila* cell lines, rat and Drosophila embryos. The cell source of each lysate is indicated above the lanes. Notch2 expression was monitored with antibody bhN6D and Notch1 expression with antibody bTAN20. Both recognize intracellular epitopes of the protein (Zagouras et al., 1995, Proc. Natl. Acad. Sci. USA 92:6414-6418). Figure 3A and 3B show Notch2 expression. Figure 3C and 3D show Notch1 expression. Figure 3E shows the expression of *Drosophila* Notch in embryos, *Drosophila* KC cultured cells, which endogenously express Notch, and *Drosophila* S2 cells, which do not endogenously express Notch but have been stably transfected with a Notch expression vector. The antibody used (9C6) recognizes an intracellular epitope (Fehon et al., 1990, Cell 61:523-534). In all the panels the 110 kDa major breakdown product (N^{TM}) and the position of the full-length Notch protein are indicated. Molecular weight markers are shown on the left of each panel.

Figure 4 shows the subcellular location of the 110 kDa (N^{TM}) fragment. Subcellular fractionation of SJ-NB5 cells followed by SDS-PAGE and Western blot with a Notch2 antibody

raised against an intracellular epitope (bhN6D). Whole cell lysate is shown on the left lane. This lysate was centrifuged at 900 x g and the pellet (0.9K) is in the second lane. This pellet was resuspended and analyzed on a sucrose step gradient at 0%, 40% and 50% sucrose. The pellet of the gradient, which contains the nuclei (NP), and the interphases are analyzed as indicated in the last three lanes. The supernatant of the initial low spin was centrifuged at 40,000 x g and the pellet was analyzed in the lane indicated as 40K. Finally the supernatant of the 40K spin was centrifuged again at 100,000 x g (lanes indicated as 100K) and the resulting pellet (P) and supernatant (S) were loaded on the gel.

Figure 5 shows that the 110 kDa (N^{TM}) fragment is expressed on the cell surface. SJ-NB5 cells were treated with biotin (+Biotin) while control cells were not (-Biotin). Each sample was lysed and divided into three equal portions precipitated with immobilized streptavidin, anti-Notch2 antibody PGHN (lanes 1, 2 and 3) or normal rabbit serum (lanes 4, 5 and 6). Samples were run on a 4-20% SDS-PAGE gel and blotted with antibody bhN6D. Molecular weight markers are shown on the left. N^{TM} accumulates on the surface, while full-length Notch is not precipitated by streptavidin.

Figures 6A-6B show that the processing of Notch2 is blocked by Brefeldin A and at 19°C. Figure 6A shows the results of a pulse labeling experiment in SJ-NB5 cells in the presence or absence of Brefeldin A. [^{35}S]-Methionine was allowed to incorporate for 20 minutes and then chased for 0, 15, 30, 45, 60, 90 minutes at 37°C. The cell lysates were immunoprecipitated by PGHN (a polyclonal antibody raised against intracellular Notch2 epitopes, Zagouras et al., 1995, Proc. Natl. Acad. Sci. USA 92:6414-6418), analyzed by SDS-PAGE and followed by fluorography. Figure 6B shows SJ-NB5 cells labeled with [^{35}S]-methionine for 20 minutes, chased either at 37°C or 19°C for 0, 30, 60, 90 minutes, immunoprecipitated by PGHN and analyzed by SDS-PAGE, followed by fluorography. Two fragments accumulate during

the chase and co-immunoprecipitate with PGHN: a 180 kDa fragment (N^{EC}) and a 110 kDa fragment (N^{TM}).

Figure 7 shows that full-length Notch does not accumulate on the cell surface. SJ-NB5 cells were pulse labeled with [^{35}S]-methionine for 10 minutes, chased for 0, 15, 30, 45, 60, 90 and 120 minutes, and this was followed by the biotinylation of the surface proteins. The cell lysates were immunoprecipitated with the polyclonal Notch2 antibody PGHN (Zagouras et al., 1995, Proc. Natl. Acad. Sci. USA 92:6414-6418). Lanes corresponding to those lysates are designated T and show all the antigens recognized by PGHN. At each time point, part of the PGHN immunoprecipitate was resuspended and then immunoprecipitated by streptavidin, which would correspond to the Notch antigens on the surface (S lanes). The immunoprecipitation products were analyzed by SDS-PAGE followed by fluorography. The accumulation of the N^{TM} and N^{EC} fragments is evident, while full-length Notch is not detected on the surface.

Figure 8 shows that Delta binds to the heterodimeric form of Notch. Identical amounts of cell lysates were precipitated with Delta antibodies from S2 cells expressing Notch (lane 1), S2 cells expressing Delta (lane 2), Notch and Delta expressing cells after one hour of aggregation (lane 3) and Notch and Delta expressing cells after two hours of aggregation (lane 4). In addition, a cell lysate of Notch expressing cells which had not been incubated with Delta antibody is shown in lane 5. All lanes are visualized with Notch antibody 9C6, which recognizes intracellular epitopes. The 110 kd Notch N^{TM} fragment is immunoprecipitated by the Delta antibodies in the extracts from Notch/Delta cell aggregates.

Figure 9 is a model for the trafficking of the Notch receptor. Full-length Notch is synthesized in the ER (N) and then cleaved in the trans-Golgi network (TGN) extracellular region, producing two fragments, N^{TM} and N^{EC}. Full-length Notch (N) reflects an inactive, presumably newly synthesized form of the receptor, which is not seen on the

surface. N^{TM} and N^{EC}, produced by a cleavage in the extracellular domain, are tethered together on the surface via a DTT-sensitive link, constituting the active form of the receptor that can interact with ligands (horizontally lined circle) and/or interact homotypically with another Notch receptor or conceivably with other surface molecules.

Figures 10A-10B are Western blot analyses showing the Notch cleavage pattern in human cells, in *Drosophila* embryo extracts and in *Drosophila* S2 cells which recombinantly express Notch. Figure 10A is a Western blot of SJ-NB5 cells (human neuroblastoma) using antibody bhN6D and Figure 10B is a Western blot of *Drosophila* embryo extracts and in *Drosophila* S2 cells which recombinantly express Notch using antibody 9C6. Molecular weight markers are indicated at left for both Figures 10A and 10B.

Figures 11A-11B show that N^{EC} and N^{TM} are associated in a non-covalent manner. Figure 11A is a Western blot analysis demonstrating that N^{EC} is present in the supernatant of Notch expressing S2 cells that have been resuspended in 2 mM EDTA, Tris-HCl saline buffer (EDTA), whereas in the presence of 2 mM $CaCl_2$ (Ca^{2+}) insignificant amounts of N^{EC} are detected. Figure 11B is a Western blot of a sucrose density centrifugation of S2 cell extracts that shows N^{EC} and N^{TM} co-sediment in the presence of $CaCl_2$, whereas N^{EC} and N^{TM} sediment separately in the presence of EDTA.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to methods for detecting or measuring Notch activation by observing or measuring the appearance of Notch on the cell surface or by observing or measuring Notch cleavage products, that are indicative of Notch activation. In one aspect of this embodiment of the invention, the method for detecting or measuring Notch activation in a cell comprises detecting or measuring the expression of Notch on the surface of said cell, wherein the presence and amount of Notch on the surface indicates the presence and amount, respectively, of Notch

activation. In another aspect, the method comprises detecting or measuring the expression of one or more Notch cleavage products selected from the group consisting of N^{EC} and N^{TM}. In yet another aspect, the method comprises detecting or measuring one or more fragments of Notch selected from the group consisting of an amino-terminal fragment of full-length Notch terminating between the epidermal growth factor-like repeat domain and the transmembrane domain (in particular, between the Lin-12/Notch repeats and the transmembrane domain) of full-length Notch, and a carboxy-terminal fragment of full-length Notch with its amino terminus situated between the epidermal growth factor-like repeat domain and the transmembrane domain (in particular, between the Lin-12/Notch repeats and the transmembrane domain), or detecting or measuring one or more fragments of Notch selected from the group consisting of Notch fragments having a molecular weight of about 270, 200, 170, 140, 110, 100, 90 and 85 kilodaltons. In yet another aspect, the method comprises detecting or measuring a Notch heterodimer containing a reducing agent-sensitive linkage, in particular, a non-covalent, metal ion-dependent (*e.g.*, calcium ion-dependent) linkage.

The present invention is based, at least in part, on the discovery that Notch in its active form, *i.e.*, the form that mediates signal transduction and that binds Notch ligands such as Delta, is a heterodimer of two Notch cleavage products, an about (±10%) 180 kilodaltons (kDa) subunit (N^{EC}) and an about (±10%) 110 kDa subunit (N^{TM}), which are tethered together through a reducing agent-sensitive linkage, in particular, a non-covalent, metal ion-dependent (*e.g.*, calcium ion-dependent) linkage. Full length Notch is not expressed on the cell surface and is ligand inaccessible. As shown by way of example *infra*, the two subunits arise due to a proteolytic cleavage of the full length Notch molecule in the trans-Golgi at a site in Notch amino-terminal to the transmembrane domain and carboxy-terminal to the EGF repeat region, thus generating an extracellular fragment (N^{EC}) of

about 180 kDa and a transmembrane/intracellular fragment (N^{TM}) of about 110 kDa. The detection of full length Notch and of Notch cleavage products, as well as Notch that is present on the cell surface, can be carried out by methods well known to those of skill in the art, *e.g.*, precipitation or binding to an immobilized binding partner (*e.g.*, on a plate or column), *e.g.*, anti-Notch antibodies or ligands of Notch, such as Delta and Serrate.

Logeat et al., 1998, Proc. Natl. Acad. Sci. USA 95:8108-8112, demonstrated that the convertase furin cleaves human Notch1 on the carboxy side of the sequence ArgGlnArgArg (amino acids 1651-1654), which is in the region between the Lin-12/Notch repeats and the transmembrane domain. Although human Notch2, as well as mouse Notch (mNotch), do not have sequence identity at this region with human Notch1, we believe the cleavage site in these proteins likely to be between the epidermal growth factor-like repeats and the transmembrane domain, *e.g.*, between the Lin-12/Notch repeats and the transmembrane domain.

The detection or measurement of Notch activation is important in the study and manipulation of differentiation processes, since Notch plays a key role in cell fate (differentiation) determination. Also, disorders of cell fate, in particular hyperproliferative (*e.g.*, cancer) or hypoproliferative disorders, involving aberrant or undesirable levels of active Notch expression can be diagnosed or screened for by detecting such active Notch expression, as described more fully *infra*. Molecules that modulate Notch function are important tools for studying and manipulating differentiation processes, *e.g.*, in expanding cell populations without substantial differentiation (International Publication WO 97/11716), in cancer studies and therapy (International Publication WO 94/07474), and differentiation studies on normal tissue.

In another embodiment, the present invention is also directed to methods for identifying a molecule that modulates Notch activation by detecting or measuring a change

in the amount of Notch expressed on the cell surface or a change in the amount or pattern of Notch cleavage products. In one aspect of this embodiment of the invention, the method for identifying a modulator of Notch activation comprises providing a cell with a candidate modulator molecule and detecting or measuring the amount of Notch on the surface of the cell, in which a difference in the presence or amount compared to a cell not contacted with the candidate molecule indicates that the candidate molecule modulates Notch activation. In another aspect, the method for identifying a modulator of Notch activation comprises providing a cell with a candidate modulator molecule and detecting or measuring the expression by the cell of one or more Notch cleavage products selected from the group consisting of N^{EC} and N^{TM}, in which a difference in the presence or amount of said one or more cleavage products compared to a Notch cell not contacted with the candidate molecule indicates that the molecule modulates Notch activity.

In an alternative aspect, the method for identifying a modulator of Notch activation comprises contacting a candidate modulator molecule with a full length Notch in the presence of a composition comprising cellular proteins, under conditions conducive to cleavage of the full-length Notch by one or more components of the composition and detecting or measuring the amount of Notch cleavage products N^{EC} and N^{TM} that result, in which a difference in the presence or amount of said Notch cleavage products compared to a full-length Notch in presence of said composition not contacted with the candidate molecule indicates that the molecule modulates Notch activity.

The present invention is also directed to a substantially purified active form of Notch which comprises Notch fragments tethered together through a reducing agent-sensitive linkage, in particular, a non-covalent, metal ion-dependent (*e.g.*, calcium ion-dependent) linkage, and pharmaceutical compositions and kits thereof.

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into subsections, as follows.

5.1 DETECTION OF THE ACTIVE FORM OF NOTCH

In this embodiment of the invention, methods are provided for the detection or measuring of Notch activation comprising detecting or measuring the expression of Notch on the surface of said cell, wherein the presence and amount of Notch on the surface indicates the presence and amount, respectively, of Notch activation, or detecting or measuring the expression of one or more Notch cleavage products selected from the group consisting of N^{EC} and N^{TM}, or detecting or measuring one or more fragments of Notch selected from the group consisting of an amino-terminal fragment of full-length Notch terminating between the epidermal growth factor-like repeat domain and the transmembrane domain (in particular, between the Lin-12/Notch repeats and the transmembrane domain) of full-length Notch repeats of full-length Notch, and a carboxy-terminal fragment with its amino terminus situated between the epidermal growth factor-like repeat domain and the transmembrane domain (in particular, between the Lin-12/Notch repeats and the transmembrane domain), or detecting or measuring one or more fragments of Notch selected from the group consisting of Notch fragments having a molecular weight of about 270, 200, 170, 140, 110, 100, 90 and 85 kilodaltons, or detecting or measuring a Notch heterodimer containing a reducing agent-sensitive linkage (particularly, a non-covalent, metal ion-dependent (*e.g.*, calcium ion-dependent) linkage), or detecting or measuring a pattern of Notch fragments such as shown in Figure 10A or 10B (with approximate molecular weights indicated on the right side of each figure). The assay methods of the invention are preferably carried out *in vitro* or in cell culture, but alternatively, may be carried out *in vivo* in an animal.

The invention is based, at least in part, on the discovery that the active form of Notch is not the full

length form but rather a cell surface expressed heterodimer consisting of N^{EC} and N^{TM} Notch fragments tethered together through a reducing agent-sensitive linkage, in particular, a non-covalent, metal ion-dependent, (*e.g.*, calcium ion-dependent) linkage.

In an alternative embodiment, methods are provided for the detecting or measuring of Notch activation comprising detecting or measuring the levels of N^{EC} in cell culture medium in the presence of an amount of a divalent metal ion chelator such as but not limited to EDTA or EGTA sufficient to cause dissociation of Notch surface heterodimers, wherein the presence and amount of Notch in the culture medium indicates the presence and amount, respectively, of Notch activation.

The ability to detect the expression of the active form of Notch is an important diagnostic/screening tool for cancer since Notch is known to be aberrantly expressed in neoplasias. For example, the aberrant expression of a truncated Notch1 RNA is seen in a human T cell leukemia (Ellison et al., 1991, Cell 66:649-661). Further, human Notch1 and Notch2 are not normally expressed in columnar epithelia but are expressed in neoplasias, in cases ranging from relatively benign squamous metaplasias to cancerous invasive adenccarcinomas in which columnar epithelia are replaced by these tumors (Mello et al., 1994, Cell 77:95-106; see also International Publication WO 94/07474). Therefore, using the assay methods of the present invention, aberrant forms or levels of Notch activation, which may be present in various malignancies, can be detected.

Any method known in the art for detecting or measuring the expression of Notch on the cell surface or the expression of Notch cleavage products indicative of Notch activation can be used. For example, and not by way of limitation, one such method of detection of the active form of Notch by detecting cell surface expression of Notch is by labeling generally the cell surface-expressed proteins with, *e.g.*, biotin or ^{125}I, and then detecting the label on Notch.

If no label is detected, Notch is not expressed on the cell surface, and thus the active form of Notch is not expressed. Another method of detection of the active form of Notch is, *e.g.*, by labeling generally the cell surface-expressed proteins, with, *e.g.*, biotin or ^{125}I, adding a sufficient amount of a divalent metal ion chelator to disrupt the interaction between N^{EC} and N^{TM}, and then detecting the label in the cell culture medium. If no label is detected, the active form of Notch is not expressed. In a specific embodiment, Notch can be isolated using, *e.g.*, an anti-Notch antibody or Notch ligand or a binding fragment of a Notch ligand, before detecting the label on Notch. A particular method of detecting cell surface Notch is to contact a labelled anti-Notch antibody, *e.g.*, labeled with a fluorescent dye or with a radioactive isotope such as ^{125}I, to whole cells and then to detect cells having the label through, *e.g.*, flow cytometry, fluorescent activated cell sorting (FACS) analysis, or scintillation counting.

Another method is to detect the active form of Notch by detecting one or more Notch cleavage products selected from the group consisting of N^{EC} and N^{TM}, or selected from the group consisting of an amino-terminal fragment of full-length Notch terminating between the epidermal growth factor-like repeat domain and the transmembrane domain (in particular, between the Lin-12/Notch repeats and the transmembrane domain) of full-length Notch, and a carboxy-terminal fragment of full-length Notch with its amino terminus situated between the epidermal growth factor-like repeat domain and the transmembrane domain (in particular, between the Lin-12/Notch repeats and the transmembrane domain), or selected from the group consisting of Notch fragments having a molecular weight of about 270, 200, 170, 140, 110, 100, 90 and 85 kilodaltons. Yet another method is to detect a pattern of Notch cleavage products as shown in Figure 10A or 10B.

Detection of such cleavage products can be done, *e.g.*, by immunoprecipitating the cleavage products with an

anti-Notch antibody or binding to anti-Notch antibody on an immunoaffinity column or immobilized on a plate or in a well, or visualizing the fragments by Western blotting. In a specific embodiment, the cleavage products can be labelled by general cell surface labeling, or, alternatively, by pulse labeling the cells by incubation in culture medium containing a radioactive label, or, alternatively, it can be anti-Notch antibody (or antibody binding partner) that is labeled rather than the Notch cleavage products.

According to a specific embodiment of the invention, antibodies and fragments containing the binding domain thereof, directed against Notch are used to detect Notch in a specific embodiment of the above methods. Accordingly, Notch proteins, fragments or analogs or derivatives thereof, in particular, human Notch proteins or fragments thereof, may be used as immunogens to generate anti-Notch protein antibodies. Such antibodies can be polyclonal, monoclonal, chimeric, single chain, Fab fragments, or from an Fab expression library. In a specific embodiment, antibodies specific to EGF-like repeats 11 and 12 of Notch may be prepared. In other embodiments, antibodies reactive with the extracellular domain of Notch can be generated. In one embodiment, antibodies specific to human Notch are produced.

Various procedures known in the art may be used for the production of polyclonal antibodies to a Notch protein or peptide. In a particular embodiment, rabbit polyclonal antibodies to an epitope of the human Notch proteins depicted in Figure 2, or a subsequence thereof, can be obtained. For the production of antibody, various host animals can be immunized by injection with the native Notch protein, or a synthetic version, or fragment thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin,

pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

For preparation of monoclonal antibodies directed toward a Notch protein sequence, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

Antibody fragments which contain the idiotype (binding domain) of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.*, ELISA (enzyme-linked immunosorbent assay). For example, to select antibodies which recognize the adhesive domain of a Notch protein, one may assay generated hybridomas for a product which binds to a protein fragment containing such domain. For selection of an antibody specific to human Notch, one can select on the basis of positive binding to human Notch and a lack of binding to *Drosophila* Notch.

Another method to detect the active form of Notch is to use a Notch ligand or other Notch binding partner or binding fragment thereof, such as Delta or Serrate and

members of the Delta/Serrate family, to bind to Notch (*e.g.*, when the ligand is labeled), or to recover Notch by coimmunoprecipitating with the appropriate anti-Notch ligand antibody to co-immunoprecipitate Notch cleavage products in the active Notch heterodimer bound to the Notch ligand, etc. Other Notch binding proteins, in addition to extracellular ligands, can also be used to co-immunoprecipitate Notch cleavage fragments. Examples of Notch ligands include but are not limited to Delta, Serrate, Deltex, and fragments and derivatives thereof that mediate binding to Notch; see *e.g.*, International Publications WO 92/19734, WO 96/27610, WO 97/01571, and WO 97/18822.

Similar procedures to those described *supra* can be used to make antibodies to domains of other proteins (particularly toporythmic proteins) that bind or otherwise interact with Notch (*e.g.*, binding fragments of Delta or Serrate).

Another method that can be used to detect the cell surface-expressed active form of Notch is to assay for cell adhesion between cells expressing Notch and cells expressing a Notch ligand, such as Delta or Serrate or members of the Delta/Serrate family, *e.g.*, according to the method disclosed in Rebay et al., 1991, Cell 67:687-699 and International Publication WO 92/19734. In one aspect, this method comprises contacting a first plurality of said cell with a second plurality of cells expressing a Notch ligand on their surfaces; and measuring cell aggregation between cells in said first plurality and cells in second plurality.

The cell in which Notch activation is detected or measured can be any cell, *e.g.*, one that endogenously or recombinantly expresses Notch. The cell can be vertebrate, insect (*e.g.*, *Drosophila*), *C. elegans*, mammalian, bovine, murine, rat, avian, fish, primate, human, etc. The Notch which is expressed can be vertebrate, insect, *C. elegans*, mammalian, bovine, murine, rat, avian, fish, primate, human, etc. The cell can be a cell of primary tissue, a cell line, or of an animal containing and expressing a Notch transgene.

For example, the transgenic animal can be a *Drosophila* (*e.g.*, melanogaster) or a *C. elegans*. In a preferred embodiment, the transgene encodes a human Notch. Transgenic animals can be made by standard methods well known in the art (*e.g.*, by use of P element transposons as a vector in *Drosophila*).

5.2 METHODS OF IDENTIFYING MODULATORS

In one embodiment of the invention, methods are provided for the identification of modulators, *e.g.*, inhibitors, antagonists, or agonists, of Notch activation by detecting the ability of the modulators to effect cleavage of full length Notch and/or its expression on the cell surface. The invention is based, at least in part, on the discovery that the active form of Notch is not the full length protein but rather a cell surface-expressed heterodimer consisting of N^{EC} and N^{TM} Notch fragments (Notch cleavage products) tethered together through a reducing agent-sensitive linkage, in particular, a non-covalent, metal-ion-dependent (*e.g.*, calcium ion-dependent) linkage. In one aspect of this embodiment of the invention, the method for identifying a modulator of Notch activation comprises providing a cell with a candidate modulator molecule and detecting or measuring the amount of Notch on the surface of the cell, in which a difference in the presence or amount compared to a cell not contacted with the candidate molecule indicates that the candidate molecule modulates Notch activation. In another aspect of this embodiment of the invention, the method comprises providing a cell with a candidate modulator molecule and detecting or measuring the expression by the cell of one or more Notch cleavage products selected from the group consisting of N^{EC} and N^{TM}, in which a difference in the presence or amount of said one or more cleavage products compared to a Notch cell not contacted with the candidate molecule indicates that the molecule modulates Notch activity. In yet another aspect, the method comprises providing a cell with a candidate modulator molecule and detecting or measuring the amount of the expression by the

cell of one or more fragments of Notch selected from the group consisting of an amino-terminal fragment of full-length Notch terminating between the epidermal growth factor-like repeat domain and the transmembrane domain (in particular, between the Lin-12/Notch repeats and the transmembrane domain) of full-length Notch, and a carboxy-terminal fragment of full-length Notch with its amino terminus situated between the epidermal growth factor-like repeat domain and the transmembrane domain (in particular, between the Lin-12/Notch repeats and the transmembrane domain); in which a difference in the presence or amount of said one or more fragments compared to a Notch cell not contacted with the candidate molecule indicates that the molecule modulates Notch activity.

In yet another aspect, the method comprises providing a cell with a candidate modulator molecule and detecting or measuring the expression by the cell of one or more fragments of Notch selected from the group consisting of Notch fragments having a molecular weight of about 270, 200, 170, 140, 110, 100, 90 and 85 kilodaltons, in which a difference in the presence or amount of said one or more fragments compared to a Notch cell not contacted with the candidate molecule indicates that the molecule modulates Notch activity. In another aspect, the method comprises providing a cell with a candidate modulator molecule and detecting or measuring the amount of the expression by the cell of a pattern of Notch cleavage products as shown in Figure 10A or 10B, in which a difference in the presence or amount of said pattern compared to a Notch cell not contacted with the candidate molecule indicates that the molecule modulates Notch activity. In yet another aspect, the method comprises providing a cell with a candidate modulator molecule and detecting or measuring the amount of the expression by the cell of one or more Notch fragments of about 180 kilodaltons and about 110 kilodaltons, respectively, in which a difference in the presence or amount of the fragments compared to a Notch cell not contacted with

the candidate molecule indicates that the molecule modulates Notch activity. In another aspect, the method for identifying a modulator of Notch activation comprises contacting a cell with a candidate modulator molecule and detecting or measuring the amount of the expression by the cell of a Notch heterodimer containing a reducing agent-sensitive linkage, in a preferred aspect, a non-covalent, metal ion-dependent (*e.g.*, calcium ion-dependent) linkage, in which a difference in the presence or amount of the heterodimer compared to a Notch cell not contacted with the candidate molecule indicates that the molecule modulates Notch activity. In a specific aspect of this embodiment of the invention, the detecting or measuring is carried out by contacting a first plurality of said cell with a second plurality of cells expressing a Notch ligand on their surfaces; and measuring cell aggregation between cells in said first plurality and cells in second plurality.

In yet another aspect of this embodiment of the invention, the method for identifying a modulator of Notch activation comprises contacting a candidate modulator molecule with a full length Notch in the presence of a composition comprising cellular proteins, under conditions conducive to cleavage of the full-length Notch by one or more components of the composition, and detecting or measuring the amount of Notch cleavage products N^{EC} and/or N^{TM} that result, in which a difference in the presence or amount of said Notch cleavage product(s) compared to a full-length Notch in presence of said composition not contacted with the candidate molecule indicates that the molecule modulates Notch activity. In another aspect, the method for identifying a modulator of Notch activation comprises contacting a candidate modulator molecule with a full length Notch in the presence of a composition comprising cellular proteins, under conditions conducive to cleavage of the full-length Notch by one or more components of the composition, and detecting or measuring one or more fragments of Notch selected from the group consisting of an amino-terminal fragment of full-length

Notch terminating between the epidermal growth factor-like repeat domain and the transmembrane domain (in particular, between the Lin-12/Notch repeats and the transmembrane domain) of full-length Notch, and a carboxy-terminal fragment of full-length Notch with its amino terminus situated between the epidermal growth factor-like repeat domain and the transmembrane domain (in particular, between the Lin-12/Notch repeats and the transmembrane domain), that result, in which a difference in the presence or amount of said one or more Notch fragments compared to a full-length Notch in presence of said composition not contacted with the candidate molecule indicates that the molecule modulates Notch activity.

In yet another aspect, the method for identifying a modulator of Notch activation comprises contacting a candidate modulator molecule with a full length Notch in the presence of a composition comprising cellular proteins, under conditions conducive to cleavage of the full-length Notch by one or more components of the composition and detecting or measuring the amount of one or more fragments of Notch selected from the group consisting of Notch fragments having a molecular weight of about 270, 200, 170, 140, 110, 100, 90 and 85 kilodaltons, that result, in which a difference in the presence or amount of said one or more Notch fragments compared to a full-length Notch in presence of said composition not contacted with the candidate molecule indicates that the molecule modulates Notch activity. In yet another aspect, the method for identifying a modulator of Notch activation comprises contacting a candidate modulator molecule with a full length Notch in the presence of a composition comprising cellular proteins, under conditions conducive to cleavage of the full-length Notch by one or more components of the composition, and detecting or measuring the amount of a pattern of Notch cleavage products as shown in Figure 10A or 10B that result, in which a difference in the presence or amount of said pattern compared to a full-length Notch in presence of said composition not contacted with the

candidate molecule indicates that the molecule modulates Notch activity.

In yet another aspect of this embodiment of the invention, the method for identifying a modulator of Notch activation comprises contacting a candidate modulator molecule with a full length Notch in the presence of a composition comprising cellular proteins, under conditions conducive to cleavage of the full-length Notch by one or more components of the composition, and detecting or measuring the amount of one or more Notch fragments of about 180 kilodaltons and about 110 kilodaltons, respectively, that result, in which a difference in the presence or amount of said one or more Notch fragments compared to a full-length Notch in presence of said composition not contacted with the candidate molecule indicates that the molecule modulates Notch activity. In yet another aspect, the method for identifying a modulator of Notch activation comprises contacting a candidate modulator molecule with a full length Notch in the presence of a composition comprising cellular proteins, under conditions conducive to cleavage of the full-length Notch by one or more components of the composition, and detecting or measuring the amount of a Notch heterodimer containing a reducing agent-sensitive linkage, in particular, a non-covalent, metal ion-dependent (*e.g.*, calcium ion-dependent) linkage, that results, in which a difference in the presence or amount of said heterodimer compared to a full-length Notch in presence of said composition not contacted with the candidate molecule indicates that the molecule modulates Notch activity.

In a specific aspect of the embodiment using a composition comprising cellular proteins, the composition comprising cellular proteins is a cell lysate made from cells which recombinantly express Notch. In another specific aspect of this embodiment, the composition comprising cellular proteins is a cell lysate made from cells which endogenously express Notch.

Detection or measurement of Notch expressed on the cell surface and/or Notch cleavage products can be carried out by methods well known in the art and/or those methods disclosed in Section 5.1, *supra*.

The cells used in the methods of this embodiment can either endogenously or recombinantly express Notch. Examples of the cell types and Notch protein that can be expressed are described in Section 5.1. Recombinant Notch expression is carried out by introducing Notch encoding nucleic acids into expression vectors and subsequently introducing the vectors into a cell to express Notch or simply introducing Notch encoding nucleic acids into a cell for expression. Nucleic acids encoding vertebrate and non-vertebrate Notch have been cloned and sequenced and their expression is well known in the art. See, for example, International Publication WO 92/19734 and U.S. Patent No. 5,648,464, which are incorporated by reference in their entirety herein; Wharton et al., 1985, Cell 43:567-581; and Coffman et al., 1990, Science 249:1438-1441. Expression can be from expression vectors or intrachromosomal.

Any method known to those of skill in the art for the insertion of Notch-encoding DNA into a vector may be used to construct expression vectors containing a chimeric gene consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinants (genetic recombination). Expression of nucleic acid sequence encoding a Notch protein may be regulated by a second nucleic acid sequence so that the Notch protein is expressed in a host transformed with the recombinant DNA molecule. For example, expression of a Notch protein may be controlled by any promoter/enhancer element known in the art. Promoters which may be used to control Notch gene expression include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3′ long terminal repeat of Rous sarcoma virus (Yamamoto, et al.,

1980, Cell:22 787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25); see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al., Nature 303:209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner, et al., 1981, Nucl. Acids Res. 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, Nature 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58; alpha 1-

antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

Many expression vectors can be used, including but not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (*e.g.*, lambda), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain may be chosen which modulates the expression of Notch, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of Notch protein may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (*e.g.*, glycosylation, cleavage) of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the Notch protein expressed. For example, expression in a bacterial system can be used to produce an unglycosylated core protein product. Expression in yeast will produce a glycosylated product. Expression in mammalian cells can be used to ensure "native" glycosylation of a mammalian Notch protein.

In the methods of the invention in which full-length Notch is incubated with compositions comprising cellular proteins (*e.g.*, cell lysates or cell fractions) in the presence of candidate cleavage (and thus Notch

activation) modulators the expression of Notch should be such that full length Notch is expressed and proteolytic cleavage of Notch is kept to a minimum such that Notch cleavage products are easily detected over any background proteolysis. There are several methods known in the art to keep proteolysis to a minimum. For example, one manner to keep Notch cleavage to a minimum is to express Notch in cells concurrently with Brefeldin A treatment. Brefeldin A has been shown to inhibit the cleavage of Notch, see Section 6.7, *infra*. Another manner to keep Notch cleavage to a minimum is to incubate Notch expressing cells at 19°C, see also Section 6.7, *infra*. Another manner is to express Notch in cells which do not contain a protease which cleaves Notch or to express Notch in an *in vitro* transcription-translation system in the presence of a protease inhibitor such as phenylmethylsulfonylfluoride (PMSF).

5.2.1 CANDIDATE MOLECULES

Any molecule known in the art can be tested for its ability to modulate Notch activation as measured by the cell surface expression of Notch or the expression of one or more of the Notch cleavage products disclosed herein. For identifying a molecule that modulates Notch activation, candidate molecules can be directly provided to a cell expressing Notch, or, in the case of candidate proteins, can be provided by providing their encoding nucleic acids under conditions in which the nucleic acids are recombinantly expressed to produce the candidate proteins within the Notch expressing cell. In an embodiment of the invention directed to the assay using full-length Notch and a composition comprising cellular proteins, candidate molecules can also be added to a composition comprising cellular proteins (whole cell lysates, membrane fraction, etc.), preferably derived from cells endogenously or recombinantly expressing Notch.

This embodiment of the invention is well suited to screen chemical libraries for molecules which modulate, *e.g.*, inhibit, antagonize, or agonize, Notch activation. The

chemical libraries can be peptide libraries, peptidomimetic libraries, other non-peptide synthetic organic libraries, etc.

Exemplary libraries are commercially available from several sources (ArQule, Tripos/PanLabs, ChemDesign, Pharmacopoeia). In some cases, these chemical libraries are generated using combinatorial strategies that encode the identity of each member of the library on a substrate to which the member compound is attached, thus allowing direct and immediate identification of a molecule that is an effective modulator. Thus, in many combinatorial approaches, the position on a plate of a compound specifies that compound's composition. Also, in one example, a single plate position may have from 1-20 chemicals that can be screened by administration to a well containing the interactions of interest. Thus, if modulation is detected, smaller and smaller pools of interacting pairs can be assayed for the modulation activity. By such methods, many candidate molecules can be screened.

Many diversity libraries suitable for use are known in the art and can be used to provide compounds to be tested according to the present invention. Alternatively, libraries can be constructed using standard methods. Chemical (synthetic) libraries, recombinant expression libraries, or polysome-based libraries are exemplary types of libraries that can be used.

The libraries can be constrained or semirigid (having some degree of structural rigidity), or linear or nonconstrained. The library can be a cDNA or genomic expression library, random peptide expression library or a chemically synthesized random peptide library, or non-peptide library. Expression libraries are introduced into the cells in which the assay occurs, where the nucleic acids of the library are expressed to produce their encoded proteins.

In one embodiment, peptide libraries that can be used in the present invention may be libraries that are chemically synthesized *in vitro*. Examples of such libraries

are given in Houghten et al., 1991, Nature 354:84-86, which describes mixtures of free hexapeptides in which the first and second residues in each peptide were individually and specifically defined; Lam et al., 1991, Nature 354:82-84, which describes a "one bead, one peptide" approach in which a solid phase split synthesis scheme produced a library of peptides in which each bead in the collection had immobilized thereon a single, random sequence of amino acid residues; Medynski, 1994, Bio/Technology 12:709-710, which describes split synthesis and T-bag synthesis methods; and Gallop et al., 1994, J. Medicinal Chemistry 37(9):1233-1251. Simply by way of other examples, a combinatorial library may be prepared for use, according to the methods of Ohlmeyer et al., 1993, Proc. Natl. Acad. Sci. USA 90:10922-10926; Erb et al., 1994, Proc. Natl. Acad. Sci. USA 91:11422-11426; Houghten et al., 1992, Biotechniques 13:412; Jayawickreme et al., 1994, Proc. Natl. Acad. Sci. USA 91:1614-1618; or Salmon et al., 1993, Proc. Natl. Acad. Sci. USA 90:11708-11712. PCT Publication No. WO 93/20242 and Brenner and Lerner, 1992, Proc. Natl. Acad. Sci. USA 89:5381-5383 describe "encoded combinatorial chemical libraries," that contain oligonucleotide identifiers for each chemical polymer library member.

Further, more general, structurally constrained, organic diversity (*e.g.*, nonpeptide) libraries, can also be used. By way of example, a benzodiazepine library (*see e.g.*, Bunin et al., 1994, Proc. Natl. Acad. Sci. USA 91:4708-4712) may be used.

Conformationally constrained libraries that can be used include but are not limited to those containing invariant cysteine residues which, in an oxidizing environment, cross-link by disulfide bonds to form cystines, modified peptides (*e.g.*, incorporating fluorine, metals, isotopic labels, are phosphorylated, etc.), peptides containing one or more non-naturally occurring amino acids, non-peptide structures, and peptides containing a significant fraction of γ-carboxyglutamic acid.

Libraries of non-peptides, *e.g.*, peptide derivatives (for example, that contain one or more non-naturally occurring amino acids) can also be used. One example of these are peptoid libraries (Simon et al., 1992, Proc. Natl. Acad. Sci. USA 89:9367-9371). Peptoids are polymers of non-natural amino acids that have naturally occurring side chains attached not to the alpha carbon but to the backbone amino nitrogen. Since peptoids are not easily degraded by human digestive enzymes, they are advantageously more easily adaptable to drug use. Another example of a library that can be used, in which the amide functionalities in peptides have been permethylated to generate a chemically transformed combinatorial library, is described by Ostresh et al., 1994, Proc. Natl. Acad. Sci. USA 91:11138-11142).

The members of the peptide libraries that can be screened according to the invention are not limited to containing the 20 naturally occurring amino acids. In particular, chemically synthesized libraries and polysome based libraries allow the use of amino acids in addition to the 20 naturally occurring amino acids (by their inclusion in the precursor pool of amino acids used in library production). In specific embodiments, the library members contain one or more non-natural or non-classical amino acids or cyclic peptides. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, α-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid; γ-Abu, ε-Ahx, 6-amino hexanoic acid; Aib, 2-amino isobutyric acid; 3-amino propionic acid; ornithine; norleucine; norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, ß-alanine, designer amino acids such as ß-methyl amino acids, Cα-methyl amino acids, Nα-methyl amino acids, fluoro-amino acids and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

Further, toporythmic proteins, derivatives and fragments thereof, can be tested for the ability to modulate

Notch activation. Toporythmic proteins, and more generally, members of the "Notch cascade" or the "Notch group" of genes, include Notch, Delta, Serrate, and other members of the Delta/Serrate family, which are identified by genetic (as detected phenotypically, *e.g.*, in *Drosophila*) or molecular interaction (*e.g.*, binding *in vitro*). See, International Publications WO 92/19734, WO 97/18822, WO 96/27610, and WO 97/01571 and references therein, for examples of vertebrate and non-vertebrate members of the Notch family of genes.

5.3 HETERODIMERIC NOTCH

The present invention is also directed to a substantially purified heterodimeric form of Notch comprising Notch fragments tethered together through a reducing agent-sensitive linkage, in particular, a non-covalent, metal ion-dependent (*e.g.*, calcium ion-dependent) linkage. In its active state Notch is a heterodimer of an about (±10%) 180 kilodaltons (kDa) subunit (N^{EC}) and an about (±10%) 110 kDa subunit (N^{TM}), which are tethered together through a reducing agent-sensitive linkage, particularly, a non-covalent, metal ion-dependent (*e.g.*, calcium ion-dependent) linkage. As shown by way of example *infra*, the two subunits arise due to a proteolytic cleavage of the full length Notch molecule in the trans-Golgi at a site in Notch amino-terminal to the transmembrane domain and carboxy-terminal to the EGF repeat region, thus generating an extracellular fragment (N^{EC}) of about 180 kDa and a transmembrane/intracellular fragment (N^{TM}) of about 110 kDa.

The present invention is also directed to an amino-terminal fragment of full-length Notch terminating between the epidermal growth factor-like repeat domain and the transmembrane domain (in particular, between the Lin-12/Notch repeats and the transmembrane domain) of full-length Notch. The present invention is also directed to a carboxy-terminal fragment of full-length Notch with its amino terminus situated between the epidermal growth factor-like repeat

domain and the transmembrane domain (in particular, between the Lin-12/Notch repeats and the transmembrane domain).

Nucleic acids encoding vertebrate and non-vertebrate Notch have been cloned and sequenced. See, for example, WO 92/19734 and U.S. Patent No. 5,648,464, which are incorporated by reference in their entirety herein; Wharton et al., 1985, Cell 43:567-581; and Coffman et al., 1990, Science 249:1438-1441. These nucleic acids can be used to express the full length Notch molecule either *in vivo* or *in vitro*, and either the full length molecule is isolated and then proteolytically cleaved (*e.g.*, by exposure to cell lysates) or the full-length Notch is physiologically cleaved by the cell and the fragment(s) are then isolated therefrom. Also, the Notch encoding nucleic acids can be subcloned to express the two subunits N^{EC} and N^{TM}, respectively, either *in vivo* or *in vitro*, which can then be isolated, and if desired, can then be tethered together by addition of, or in the presence of, Ca^{2+} to form a non-covalent, metal ion-dependent, reducing agent-sensitive linkage.

The present invention is also directed to pharmaceutical compositions comprising the heterodimeric form of Notch, or the amino-terminal fragment, or the carboxy-terminal fragment, or mixtures thereof suitable for *in vivo* administration, in combination with a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The carrier and composition can be sterile. The formulation should suit the mode of administration.

The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol,

lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

6. INTRACELLULAR CLEAVAGE OF NOTCH LEADS TO A HETERODIMERIC RECEPTOR ON THE PLASMA MEMBRANE

Previous models for signal transduction via the Notch pathway have depicted the full-length Notch receptor expressed at the cell surface. Evidence is presented herein demonstrating that the Notch receptor on the plasma membrane

is cleaved. This cleavage is an evolutionary conserved, general property of Notch and occurs in the trans-Golgi network as the receptor traffics towards the plasma membrane. Although full-length Notch is detectable in the cell, it does not reach the surface. Cleavage results in a C-terminal fragment, N^{TM}, which appears to be cleaved N-terminal to the transmembrane domain, and an N-terminal fragment N^{EC} that contains most of the extracellular region. Evidence is provided herein that these fragments are tethered together on the plasma membrane by a link that is sensitive to reducing conditions and dependent upon the presence of metal ions, forming a heterodimeric receptor. On the basis of the experimental evidence gathered, it is proposed that the active, ligand accessible form of the receptor is the heterodimeric form, whereas full-length Notch reflects newly synthesized, intracellular and, hence, inactive molecules.

6.1 MATERIALS AND METHODS

6.1.1 ISOLATING AND SEQUENCING HUMAN NOTCH2 cDNAs

A human fetal brain cDNA Zap II library (from 17-18 week embryo; Stratagene, La Jolla, CA.) was used in the screening for human *Notch* homologs. The Notch cDNA clones were originally obtained by using a probe encoding portions of the human Notch2 protein (*hN2K* and *hN5K*), (Stifani et al., 1992, Nature Genetics 2:119-127). A probe used to screen for cDNAs spanning 5′ regions of the human *Notch2* gene was generated from the *hN2K* cDNA. Because the extreme 5′ terminus of the human *Notch2* gene was not isolated using this probe, advantage was taken of the fortuitous isolation of a human *Notch2* cDNA (Adams, et al., 1993, Nature Genetics 4:256-267) that extends further 5′, as determined by sequence comparison to the rat *Notch2* cDNA isolated by Weinmaster et al., 1992, Development 116:931-941. Although this human cDNA does not extend to the extreme 5′ end of the human *Notch2* coding region, it was used to generate a new probe that was closer to the 5′ end of the gene. This probe was used to

isolate the 5'-most cDNAs encoding human *Notch2*. Sequencing was done using the Sequenase™ Kit (United States Biochemical Corporation, Cleveland, OH).

6.1.2 CELL CULTURE

Human neuroblastoma (SJ-NB5) cells were grown at 37°C in an atmosphere of 5% CO_2/95% air, in RPMI (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY), 2 mM L-Glutamine (ICN Biomedicals, Inc., Costa Mesa, CA), 100 μg/ml penicillin, and 100 μg/ml streptomycin (ICN Biomedicals, Inc., Costa Mesa, CA). Cells were dissociated using phosphate buffered saline (PBS) with 0.25% trypsin and 0.03% EDTA (J.T. Baker, Inc., Phillipsburg, NJ), and subcultured at ratios of 1:3 to 1:10. HaCat Cells (cultured human keratinocytes) were a gift from Dr. Michael Reiss (Yale University). Aggregation experiments and the maintenance of *Drosophila* S2 and KC cells were as described in Fehon et al., 1990, Cell 61:523-534.

6.1.3 ANTIBODIES

Antibodies bhN6D and bTAN20 are monoclonal antibodies (rat, IgG) directed against the non-conserved intracellular epitopes of human Notch2 and Notch1, respectively (Zagouras et al., 1995, Proc. Natl. Acad. Sci. USA 92:6414-6418). On western blots they recognize specifically Notch1 and Notch2 but are not useful for immunoprecipitations. In contrast, antibody PGHN, a polyclonal antibody (Rabbit, IgG) directed against intracellular epitopes of human Notch2, can be used to immunoprecipitate Notch2 (Zagouras et al., 1995, Proc. Natl. Acad. Sci. USA 92:6414-6418). The *Drosophila* antibody 9C6 is a monoclonal antibody which recognizes intracellular epitopes of Notch (Fehon et al., 1990, Cell 61:523-534).

6.1.4 SUBCELLULAR FRACTIONATION AND WESTERN BLOTTING

SJ-NB5 cells were grown to 80-90% confluence in six T-75 tissue culture flasks, scraped in TBS, washed once and resuspended in 1 ml cold buffer A (75 mM KCl; 10 mM imidazole, pH 7.2; 1 mM EGTA; 2.5 mM $MgCl_2$; 0.02% NaN_3; 1 mM DTT; and 1 mM Pefabloc SC [Boehringer Mannheim]). During the fractionation process all samples were kept on ice and resuspended in cold buffer A. Pellet samples at all stages of fractionation were resuspended in their original volumes so that stoichiometric ratios of all samples would be equivalent.

Cells were homogenized using Omini's hand homogenizer with microscopic monitoring of cell lysis throughout homogenization. A 50 μl aliquot was kept as Fraction 1 (whole cell lysate). The lysate was then centrifuged at low speed (900 x g) for 5 minutes at 4°C. The resulting pellet was resuspended in buffer A, with a 50 μl aliquot of the suspension as Fraction 2 (0.9K/P). The suspension was centrifuged again. The pellet was washed once with buffer A. After a third centrifugation, the pellet was resuspended in 200 μl buffer A, mixed with 1.8 ml 60% sucrose made in buffer A containing 5 mM $MgCl_2$, and then transferred to a Beckman SW 50.1 centrifuge tube. The suspension was overlaid with 2 ml 40% sucrose-buffer A, and then 2 ml buffer A. The sample was centrifuged at 100,000 x g for 1 hr at 4°C. Two banded fractions were collected separately and 50 μl aliquots were kept. The upper and lower fractions were termed 40/0 and 40/50, respectively, and the nuclear pellet at bottom was resuspended in buffer A and designated NP.

The supernatant from the 900 x g spin was centrifuged again at 40,000 x g for 15 minutes at 4°C using a Sorval SS-34 fixed angle rotor. The pellet from this mid-speed spin was resuspended in buffer A and designated 40K/P. The supernatant from the mid-speed spin was further centrifuged at 100,000 x g for 1 hr at 4°C using a Beckman 70 Ti fixed angle rotor. The pellet was again resuspended in

buffer A and termed 100K/P. The supernatant was labeled 100K/S.

All samples were resuspended in 10x sample buffer, boiled and subjected to 4-20% SDS-PAGE, transferred to nitrocellulose and western blotted as described in Stifani et al., 1992, Nature Genetics 2:119-127. For western blotting, a culture supernatant of anti-human Notch2 antibody bhN6D, which recognizes the intracellular domain of human Notch2, was used at a dilution of 1:10.

6.1.5 BIOTINYLATION

Cells were grown in 10 cm plastic tissue culture plates to ~80% confluence. Six plates were used per sample (+ or - biotin). Cells were washed four times with cold PBS/CMG (PBS/0.1 mM $CaCl_2$/1.0 mM $MgCl_2$/1.0 % glucose/pH ~ 8.0). 1.7 ml of fresh, cold PBS/CMG +/- Sulfo-NHS-biotin was added to each plate, then incubated at 4°C for 15 minutes with shaking. This solution was replaced with cold RPMI without serum to absorb excess biotin, and cells were pipetted off the plates in cold serum free RPMI medium and incubated at 4°C for 15 minutes. The cells were washed three times in cold PBS/CMG solution, and were then lysed in 1.2 ml lysis buffer per sample as described in Zagouras et al., 1995, Proc. Natl. Acad. Sci. USA 92:6414-6418. After addition of SDS to 0.2%, the samples were divided into three equal portions (~400 μl each) for precipitation: 20 μl immobilized streptavidin (Immunopure Immobilized Streptavidin, Pierce, Rockford, IL); 2 μl anti-human Notch2 antibody PGHN (as described in Zagouras et al., 1995, Proc. Natl. Acad. Sci. USA 92:6414-6418), or 2 μl normal rabbit serum (NRbS) as a negative control. Samples were incubated overnight at 4°C. Staphylococcus aureus (Sigma Chemical Co., St. Louis, MO) was added to PGHN and NRbS samples at 80 μl per sample and incubations continued at 4°C for 30 minutes. All samples were washed two times in 500 μl RIPA buffer A (10 mM Tris-HCl, pH 7.4/1% Triton X-100/0.1% SDS/1% Sodium Deoxycholate/150 mM NaCl) with 2.5 ug/ml antipain (Sigma

Chemical Co., St. Louis, MO), 2.5 μg/ml aprotinin (Sigma Chemical Co., St. Louis, MO), 2 μM leupeptin (Sigma Chemical Co., St. Louis, MO), 2.5 μg/ml pepstatin (Sigma Chemical Co., St. Louis, MO), and 1 mM mg/ml phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO). Samples were resuspended in 2X sample buffer, boiled, and subjected to SDS-polyacrylamide gel electrophoresis.

6.1.6 PULSE CHASE AND BREFELDIN A TREATMENT

SJ-NB5 cells were grown on 60 mm petri dishes until ~80% confluent, washed once with PBS, and then incubated in methionine and cysteine free DMEM medium for 1 hr. 100 μCi ^{35}S-translabled Met-Cys (ICN) was added to each plate, pulsed at 37°C for 20 minutes, and chased for varying times and temperatures. The chase began by adding 2X volume complete medium plus 100 μg/ml cold methionine and cysteine to the plates.

For Brefeldin A samples, Brefeldin A was maintained at a final concentration of 10 μg/ml in starvation medium as well as in both pulse and chase. Cells were washed with cold PBS and lysed in lysis buffer (Zagouras et al., 1995, Proc. Natl. Acad. Sci. USA 92:6414-6418) containing 1 mM Pefabloc SC, 0.7 μg/ml pepstatin A, and 0.5 μg/ml leupeptin. Cell lysates were centrifuged at 14,000 rpm for 5 minutes. The supernatants were transferred to fresh tubes and pre-cleared by incubating with 5 μl normal rabbit serum and 50 μl 10% protein A-sephrose CL-4B (Pharmacia LKB) for 1 hr at 4°C. The beads were pelleted by centrifugation and the supernatants were divided into two equal aliquots. One aliquot was immunoprecipitated by incubating with rabbit polyclonal anti-human Notch2 antibody PGHN and 50 μl protein A-sephrose CL-4B for 2-3 hrs or overnight at 4°C. The other aliquot was immunoprecipitated by normal rabbit serum as control. The beads were washed three times in RIPA buffer B (150 mM NaCl, 1% NP40, 0.5% DOC, 0.1% SDS, 50 mM Tris, pH 7.5); washed once in 50 mM Tris-Cl, 150 mM NaCl, pH 7.5;

resuspended in 50 μl SDS-sample buffer; boiled; and subjected to a 3-15% gradient SDS-PAGE gel. The gel was fixed in 25% iso-propanol, 10% acetic acid for 30 minutes, soaked in Amplify™ (Amersham) for 15-30 minutes, dried, and exposed to X-ray film at -70°C.

6.1.7 PULSE CHASE AND BIOTINYLATION

SJ-NB5 cells were grown on 100 mm petri dishes until ~80% confluent and pulse chased as described in Section 6.1.6 above. The pulse-chase times shown are described in the description of the figures. After pulse-chase, the plates were put on ice, washed three times in cold PBS (containing 0.1 mM $CaCl_2$, 1 mM $MgCl_2$), and then incubated 30 minutes at 4°C in 2 ml biotinylation buffer (10 mM triethanolamine, pH 9.0, 2 mM $CaCl_2$, and 150 mM NaCl) containing 1 mg/ml NHS-SS-Biotin (Pierce, Rockford, IL) (freshly diluted from a 200 mg/ml DMSO stock stored at -20°C) with very gentle shaking, and subsequently incubated in PBS-CMG buffer (0.1 mM$CaCl_2$, 1 mM $MgCl_2$, 100 mM Glycine) for another 30 minutes to quench unreacted biotin. Post incubation plates were washed twice in PBS-CM buffer to wash away the quenched biotin. Finally, the cells were lysed and immunoprecipitated by PGHN as previously described. After the final wash, the beads were divided equally into two aliquots. One aliquot was boiled in SDS-sample buffer, the second aliquot was incubated in 100 μl elution buffer (1% SDS, 50 mM Tris-Cl, 150 mM NaCl, pH 7.5) at 80°C for 10 minutes, then 900 μl of lysis buffer was added to the eluted protein. After centrifugation, the supernatant was transferred to a fresh tube containing 50 μl of packed streptavidin beads (Pierce, Rockford, IL), and incubated at 4°C for 2-3 hrs. The beads were washed and boiled in SDS-sample buffer as described above. The samples were analyzed by 3-15% SDS-PAGE electrophoresis. The gel was fixed in 25% iso-propanol, 10% acetic acid for 30 minutes, soaked in Amplify (Amersham) for 15-30 minutes, dried, and exposed to X-ray film at -70°C.

6.2 CHARACTERIZATION OF THE HUMAN NOTCH2 GENE

The full-length cDNA encoding the human Notch2 protein is 7.8 kb in length, and the predicted protein product is 2471 amino acids long. This protein has all of the expected domains of Notch family proteins and is 92% identical to the rat Notch2 amino acid sequence overall. An amino acid alignment of human Notch2 (SEQ ID NO:1) with human Notch1 (SEQ ID NO:2), *Xenopus* Notch (Xotch) (SEQ ID NO:3) and *Drosophila* Notch (SEQ ID NO:4) is shown in Figure 2.

36 EGF repeats are present in all of the proteins shown, and each is more closely related to the corresponding EGF repeat in the other Notch homologs than to neighboring EGF repeats within the same protein. The overall identity for the EGF repeat region between the human Notch paralogs is 59%, while the identity levels between the *Drosophila* and human proteins in this region are slightly lower (51% for human Notch1 and 52% for human Notch2). While the overall amino acid conservation across the EGF repeat domain is low, the conservation of individual EGF repeats from one protein to another is variable (M. Baron and S. Artavanis-Tsakonas, unpublished results). Certain repeats, including numbers 11 and 12, which are capable of ligand-binding (Rebay et al., 1991, Cell 67:687-699), are more highly conserved than others. The overall conservation of the LN repeats is similar to that for the EGF repeats, having 54% identity between the human homologs and slightly lower values between *Drosophila* Notch and either human Notch1 or human Notch2 (49% and 44%, respectively).

In Notch2, the conservation of the intracellular domain is high. All of the known structural hallmarks of the Notch proteins are maintained, including the Ankyrin repeats, the PEST-containing region, and the basic stretch of amino acids which can function as nuclear localization signals and target truncated forms of the protein into the nucleus (Stifani et al., 1992, Nature Genetics 2:119-127; Lieber et al., 1993, Genes and Development 7:1949-1965).

6.3 THE NOTCH2 PROTEIN IS CLEAVED

Antibodies raised specifically against the human Notch2 protein were used to study its expression in cultured cells (Antibody bhN6D). Western blotting of Notch2 protein from the human SJ-NB5 neuroblastoma cell line revealed the presence of an approximately 110 kD (N^{TM}) polypeptide in addition to the full-length 300 kD protein (Figure 3A, lane 1). This lower molecular weight polypeptide is the predominant species recognized by the antibody used in this experiment. A similar processing pattern is seen in HaCat cells (Figure 3A, lane 2), a human keratinocyte cell line. The observed processing pattern is not confined to cell lines. The predominant polypeptide species recognized by the same antibody in rat embryo extracts and in a variety of human tissue extracts is also the 110 kD Notch breakdown product (Figure 3A, lane 3, and Figure 3B). Note that this western blot analysis reveals differences in the relative ratio of the full-length protein and the N^{TM} derivative among the examined tissues.

6.4 CLEAVAGE IS A GENERAL PROPERTY OF THE NOTCH RECEPTOR

Whether the characteristic cleavage pattern of the human Notch2 paralog is peculiar only to this molecule or whether it reflects a general pattern for the Notch receptor family was examined. Western blot analysis, using an antibody raised against the human Notch1 paralog (antibody bTAN20), demonstrates that this protein displays a processing pattern that is similar to that of Notch2 (Figure 3C and Figure 3D). These results are compatible with earlier analyses involving Notch1. The existence of a prominent approximately 120 kD fragment was previously demonstrated in extracts of two different human cell lines that express the Notch1 paralog (Aster et al., 1994, Cold Spring Harbor Symposia on Quantitative Biology 59:125-136). When a Notch1 expression plasmid is transfected into a baby hamster kidney cell line (BHK cells), the major Notch peptide detected in

these cells by western blot analysis is a 110 kD species (data not shown, and Zagouras et al., 1995, Proc. Natl. Acad. Sci. USA 92:6414-6418).

In order to determine whether the processing pattern seen for Notch1 and Notch2 is specific to mammalian Notch proteins, western blotting of *Drosophila* cell lysates was performed, using an antibody raised against intracellular epitopes of *Drosophila* Notch (Figure 3E; Fehon et al., 1990, Cell 61:523-534). In an embryonic extract, in addition to the clearly detectable full-length protein, several smaller Notch polypeptides, including an approximately N^{TM} band, are visible. In the KC cell line, which expresses Notch endogenously, N^{TM} is clearly detectable. Finally, in an S2 cell line, which does not express endogenous Notch but has been stably transfected with a Notch expression plasmid, N^{TM} is also prominent. It is concluded that the processing of the Notch receptor is a general property of the Notch proteins.

6.5 N^{TM} IS ASSOCIATED WITH MEMBRANES

The subcellular localization of the Notch polypeptides was determined by cell fractionation. SJ-NB5 cells were fractionated as described in Section 6.1 and the resulting fractions were examined by western blotting. Figure 4 shows a fractionation experiment in which the N^{TM} Notch fragment is associated with membrane lanes. Each fraction was also tested for the presence of syntaxin, a plasma membrane protein expressed in the same cell line (Bennett et al., 1992, Science 257:255-259). In order to ensure that such fractionation pattern is not confined to the SJ-NB5 cell line, HaCat cells and *Drosophila* S2 cells that were stably transfected with a Notch expression plasmid were fractionated (data not shown) and similar results were obtained.

6.6 THE NOTCH RECEPTOR PRESENTED AT THE CELL SURFACE IS CLEAVED

The association of the N^{TM} Notch fragment with the plasma membrane was further examined by biotin labeling of live SJ-NB5 cells (Figure 5). Biotin labeling of surface proteins was performed by incubating live cells on ice in medium containing biotin (control cells were treated with the same medium lacking biotin). The cells were subsequently lysed and divided into three equal portions that were incubated with the following reagents: (1) immobilized streptavidin, which precipitates only biotin-labeled proteins, (2) the anti-Notch2 antibody PGHN, a polyclonal antibody which recognizes an intracellular epitope and immunoprecipitates human Notch2 (Zagouras et al., 1995, Proc. Natl. Acad. Sci. USA 92:6414-6418), and (3) normal rabbit serum (NRbS). Western blotting of the precipitated products was performed using the anti-Notch2 antibody, bhN6D. The results of this experiment are shown in Figure 5. The only Notch2-related surface protein that was detected is the N^{TM} breakdown product. Immobilized streptavidin precipitated only the N^{TM} product in the biotin-labeled samples (lane 1) and no protein in the unlabeled samples (lane 4). In contrast, anti-Notch2 antibody PGHN efficiently precipitated both the full-length and breakdown Notch2 products in biotinylated (lane 2) and non-biotinylated samples (lane 5). As expected, the negative control, NRbS, does not precipitate either protein form (lanes 3 and 6).

Based on the above results it is concluded that the N^{TM} fragment is a transmembrane Notch polypeptide that resides on the plasma membrane and must be the result of a cleavage at a site in the extracellular domain.

6.7 NOTCH IS CLEAVED IN THE TRANS-GOLGI NETWORK BEFORE REACHING THE SURFACE

The experiments described above demonstrate that the steady state form of the Notch receptor found at the cell surface is a cleaved form. In an attempt to determine the

cellular compartment where Notch is cleaved, pulse labeling analyses were carried out in the presence of drugs that are known to interfere with cellular trafficking. Figure 6A demonstrates that Brefeldin A, which blocks transport between the cis- and trans-Golgi network, effectively blocks the breakdown of full-length Notch. In contrast, monensin or chloraquinone do not affect processing (data not shown). Cleavage is also effectively blocked at 19°C, a characteristic feature of processing events that occur in the trans-Golgi network (Figure 6B).

6.8 THE CLEAVED EXTRACELLULAR DOMAIN OF NOTCH IS TETHERED TO THE N^{TM} TRANSMEMBRANE FRAGMENT

In the aforementioned pulse labeling experiments (Figure 6), the accumulation of the N^{TM} fragment is closely paralleled by the accumulation of a larger fragment that is approximately 180 kD in molecular weight. This larger fragment is co-immunoprecipitated by the antibody PGHN, which recognizes an intracellular epitope of human Notch2. However, blotting of the same immunoprecipitate by western blot, using antibody bhN6D, also raised against an intracellular epitope, detects only the N^{TM} fragment.

A single cleavage of the Notch protein that produces a 110 kD fragment would also generate a second fragment of approximately 180 kD. It was therefore presumed that the N^{EC} fragment, which accumulates with kinetics indistinguishable from those of N^{TM}, corresponds to the cleaved extracellular domain of the Notch2 protein that remains attached to the N^{TM} polypeptide by a SDS and/or DTT sensitive linkage. Antibodies recognizing extracellular epitopes were not possessed by us for western blot analysis. However, the relatedness of these fragments is also supported by the fact that the appearance of N^{EC} is not inhibited by monensin or chloraquinone (data not shown) but is inhibited by Brefeldin A and a 19°C block (Figure 6). Additional supporting evidence comes from pulse labeling experiments

done with a cysteine rather than a methionine label. Labeling with cysteine shows that the N^{EC} band incorporates nearly an order of magnitude more label than the N^{TM} band, consistent with the hypothesis that it carries most of the Notch extracellular domain (data not shown).

Additional biochemical data shows that the tethering of N^{EC} to N^{TM} is not only reducing agent-sensitive but is also metal ion-dependent. Figure 11A is a Western blot analysis demonstrating that N^{EC} is present in the supernatant of Notch expressing S2 cells that have been resuspended in 2 mM EDTA, Tris-HCl saline buffer (EDTA), whereas in the presence of 2 mM $CaCl_2$ (Ca^{2+}) insignificant amounts of N^{EC} are detected. Briefly, *Drosophila* S2 cells were induced to express Notch with the addition of 0.7 mM $CuSO_4$ for 16 hours; Notch expression was under the control of the metallothionien promoter (Fehon et al., 1990, Cell 61:523-534). The cells were washed once with 20 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$ (TBS/Ca^{2+}) and were resuspended in TBS/Ca^{2+} or in 20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA (TBS/EDTA) and incubated for one hour at room temperature under slow rocking. The cells were centrifuged and pelleted and the supernatants were collected for SDS-PAGE and Western blot analysis. The blot was probed with a monoclonal antibody directed to the extracellular domain of Notch, specifically against EGF-like repeats 5-7 of *Drosophila* Notch (clone C461.3B). This monoclonal was detected using goat anti-mouse horseradish peroxidase secondary antibody and chemiluminescent substrate.

Figure 11B is a Western blot of a sucrose density centrifugation of S2 cell extracts that shows N^{EC} and N^{TM} co-sediment in the presence of $CaCl_2$, whereas N^{EC} and N^{TM} sediment separately in the presence of EDTA. Briefly, S2 expressing Notch cell extracts were prepared in either TBS/Ca^{2+} or TBS/EDTA with 1% Triton X-100, a non-ionic detergent, 1 mM PMSF, Pepstatin and Aprotinin each at 2 μg/mL and 1.8 μM Leupeptin. The extracts were sedimented through a 5-20% sucrose gradient in the above-respective buffers (without the

protease inhibitors) in a Beckman SW50.1 rotor at 34,000 rpm for 16 hours at 4°C. Fractions were collected and precipitated with 10% trichloroacetic acid. The protein pellets were resuspended in SDS-PAGE sample buffer and analyzed by SDS-PAGE and Western blotting. Blots were probed with a mixture of monoclonal antibodies C461.3B and 9C6 (directed against the intracellular domain of Notch) and detected as described above.

These data demonstrate that N^{EC} and N^{TM} dissociate in the presence of calcium ion chelators such as EDTA and EGTA. Other evidence (not shown) shows that dissociated N^{EC} and N^{TM} can reassociate upon addition of calcium. Moreover, the interaction is sensitive to reducing agents such as β-mercaptoethanol and dithiothreitol (DTT), which are likely to act by disrupting the intra-chain disulfide bonds necessary to provide the secondary structure necessary for the Notch inter-chain interactions.

These data demonstrate that N^{EC} and N^{TM} are tethered through a non-covalent, metal ion-dependent, reducing agent-sensitive linkage, not through a disulfide bridge. Although the interaction between N^{EC} and N^{TM} does not appear to be dependent on inter-chain disulfide bond(s), intra-chain disulfide bond(s) appear to play a role in maintaining secondary structure such that N^{EC} and N^{TM} are able to interact.

6.9 FULL LENGTH NOTCH DOES NOT REACH THE CELL SURFACE

The western blot analyses revealing the existence of the N^{TM} Notch fragment (Figure 3) also show varying amounts of full-length Notch. Therefore, the fate of the full-length molecule was explored by testing its expression at the cell surface.

SJ-NB5 cells were labeled with [^{35}S]-methionine for 10 minutes and then chased for varying periods. The live cells were incubated with Biotin as described above, subsequently lysed, and immunoprecipitated with PGHN. The immunoprecipitate was divided into two equal portions, one of

which was re-precipitated with immobilized streptavidin. The two sets of samples were then examined by SDS gel electrophoreses followed by fluorography. Figure 7 shows that negligible amounts of full-length Notch are detected on the surface throughout the chase, while substantial amounts of full-length molecules are precipitated by the Notch antibody (total cellular Notch). As the full-length, newly synthesized Notch decreases during the chase, the N^{TM} fragment begins to accumulate in the streptavidin precipitated reaction. N^{TM} accumulation is paralleled by the appearance of the N^{EC} fragment, consistent with the contention that this fragment represents the extracellular domain of Notch and is tethered to the N^{TM} Notch polypeptide. It is concluded that Notch protein reaches the surface in a cleaved form and that newly synthesized full-length Notch is not found on the plasma membrane.

6.10 NOTCH HETERODIMERS BIND THE LIGAND DELTA

The biological significance of the heterodimeric Notch form would be questionable if it could not bind ligands. Physical interaction between the extracellular domains of Notch and Delta have been demonstrated with the help of aggregation assays involving Delta and Notch expressing cells. If the heterodimeric form interacts with Delta after aggregation then the 110 kd N^{TM} fragment should co-immunoprecipitate using Delta antibodies. It was found that after aggregation, Delta antibodies are capable of efficiently immunoprecipitating the N^{TM} fragment demonstrating that the heterodimeric form can bind Delta (Figure 8). As expected, if the aggregation is disrupted by depleting calcium from the medium by EGTA (Fehon et al., 1993, Cell 61:523-534). Delta antibodies fail to efficiently precipitate N^{TM} (data not shown).

6.11 DISCUSSION

The strong structural conservation among both the *Drosophila* and vertebrate *Notch* gene products, and among

homologs of other components of the same pathway, imply that the molecular and biochemical mechanisms involved in Notch signaling are conserved across species boundaries. The question of what particular roles are played by the assortment of paralogs within the Notch superfamily, in combination with the various paralogs of the other pathway components, remains unclear. Expression pattern comparisons, structural similarities and the available functional data for distinct paralogs suggest that these molecules possess different expression profiles but similar biochemical and developmental properties.

It has been found that the human Notch2 protein is a highly conserved member of the Notch protein family. Specific Notch EGF repeats have been implicated in protein interactions, and missense mutations in both *Drosophila* and humans have been associated with mutant phenotypes (Hartley et al., 1987, EMBO J. 6:3407-3417; Kelley et al., 1987, Cell 51:539-548; Rebay et al., 1991, Cell 67:687-699; Joutel et al., 1996, Nature 383:707-711). Functional data regarding the cysteine rich LN repeats are lacking. Nevertheless, all Notch homologs, from flies to humans, share similar LN repeat stretches in the equivalent extracellular region of the receptor. Within the intracellular domain of the Notch proteins, all six of the Ankyrin repeats are highly conserved. These repeats have been shown to play a crucial role in Notch signaling and have been implicated in molecular interactions between *Drosophila* Notch and the Deltex protein, which behaves as a positive regulator of Notch activity (Matsuno et al., 1995, Development 121:2633-2644), and with the downstream effector Suppressor of Hairless (Fortini and Artavanis-Tsakonas, 1994, Cell 79:273-282; Matsuno et al., 1995, Development 121:2633-2644). Consistent with the high degree of conservation, Notch2 Ankyrin repeats were found to interact both with *Drosophila* as well as with human Deltex (K. Matsuno and S. Artavanis-Tsakonas, unpublished observations).

The biochemical evidence presented herein shows that the Notch receptor is cleaved in the trans-Golgi network before reaching the cell surface. Pulse labeling experiments in combination with the biotinylation data indicate that the full-length Notch molecule does not reach the plasma membrane. The varying amounts of full-length Notch detected in different cell extracts presumably reflects a newly synthesized, inactive receptor that has not yet reached the Golgi and is inaccessible to ligands. Several lines of evidence indicate that this cleavage is a general property of cellular Notch. First, the same cleavage pattern is seen in all human cell lines and human tissues examined. Second, both Notch1 and Notch2 are processed in the same way. Third, the cleavage product N^{TM} is seen in both freshly prepared embryonic rat tissues as well as in *Drosophila* extracts.

The subcellular fractionation and biotinylation studies demonstrate that N^{TM} is associated with the plasma membrane, indicating a cleavage in the extracellular region of Notch. The epitope recognized by the antibodies used here was raised against the least conserved region of the intracellular domain mapping between the PEST sequence and the Ankyrin repeats (Zagouras et al., 1995, Proc. Natl. Acad. Sci. USA 92:6414-6418). Hence, N^{TM} must include the intracellular Notch2 sequences mapping between this C-terminal epitope and the transmembrane domain. It is likely the *C. elegans* Notch-like receptors lin-12 and glp-1 are cleaved in an analogous fashion, since N-terminal and C-terminal fragments of glp-1 were found to co-purify (Crittenden et al., 1994, Development 120:2901-2911). A deletion analysis involving Notch1 expression constructs transfected in cell lines by Aster et al., 1994, Cold Spring Harbor Symposia on Quantitative Biology 59:125-136, led them to suggest that, under their experimental conditions, Notch1 may be cleaved between the LN repeats and the transmembrane domain. It is noted that in the extracts examined herein, the main Notch1 Notch processing product in SJ-NB5 and HaCat cells is, as in Notch2, approximately 110 kD. In rat

embryos, however, the main cleavage product appears to be larger. The significance of such qualitative differences in the processing pattern, or the additional breakdown products detected in our western blots, remains to be determined.

The accumulated evidence strongly indicates that N^{EC} contains the cleaved extracellular sequences of Notch, even though the lack of appropriate antibodies prevents one from directly demonstrating this hypothesis. The kinetics of N^{EC} accumulation and its inhibition profile are identical to N^{TM}. The molecular weights of the Notch breakdown products, as argued above, are also compatible with such notion. Finally, the relative incorporation of radioactive cysteine in the two fragments reflects the approximately 10:1 ratio predicted by the amino acid composition of two fragments produced by a cleavage such that N^{EC} has most of the extracellular domain. In this regard, it is noteworthy that extracellular Notch fragments are present in the conditioned medium of *Drosophila* cell cultures that express Notch (Rebay, 1993, Ph.D. Thesis Yale University; I. Rebay, R. Fehon and S. Artavanis-Tsakonas, unpublished observations). Immunocytochemical studies with *Drosophila* tissues do not reveal differences in the cellular distribution of the intracellular vs. the extracellular domain of Notch (R. Fehon and S. Artavanis-Tsakonas, unpublished observations).

The co-precipitation of the N^{EC} fragment together with N^{TM}, and the simultaneous appearance of the two fragments on the plasma membrane, indicate that N^{EC} and N^{TM} are tethered to one another. The inability to detect full-length Notch on the surface indicates that the cleaved form is the active form of the receptor.

Tethering of N^{TM} to N^{EC} is compatible with both the assumed mode of action of Notch, which necessitates interactions between the extracellular domains of the Notch receptor and its ligands, and with the cell autonomous nature of Notch signaling (Stern and Tokunaga, 1968, Proc. Natl. Acad. Sci. USA 60:1252-1259; Markopoulou et al., 1990, Journal of Experimental Zoology 27:23-27; Hoppe and

Greenspan, 1990, Development 109:875-885; Heitzler and Simpson, 1991, Cell 64:1083-1092). On the other hand, any model of Notch biochemical activity and cellular function must take into account that Notch is cleaved. Several questions raised by this finding are worth pointing out. The possibility that N^{EC} may be released from the surface, acting as an inhibitor of the pathway, must be further examined, especially in view of reports that have appeared in the literature over the years suggesting that Notch may have non-autonomous activities (Gehring, 1973, In Genetic Mechanisms of Development: The 31st Symposium of the Society for Developmental Biology. (New York: Academic Press Inc.); Technau et al., 1987, Proc. Natl. Acad. Sci. USA:84, 4500-4504; Baker and Schubiger, 1996, Development 122:617-626). Such a scenario must take into account that the expression of truncated forms of Notch, approximately corresponding to the postulated structure of N^{TM}, results in the constitutive activation of the receptor (Ellisen et al., 1991, Cell 66:649-661; Kopan et al., 1994, Development 120:2385-2396; Jennings et al., 1994, Development 120:3537-3548; Sun and Artavanis-Tsakonas, 1996, Development 122:2465-2474).

The notion that alterations in the extracellular domain may facilitate signaling events has been proposed on the basis of studies involving the expression of engineered constructs in cultured cells (Kopan et al., 1996, Proc. Natl. Acad. Sci. USA 93:1683-1688). Irrespective of how well these studies reflect the *in vivo* situation, together with the well documented *in vivo* action of truncated forms of Notch, they do raise the possibility that a ligand-dependent degradation or cleavage of the extracellular domain may result in the activation of the receptor. However, it seems unlikely that signaling would involve a simple ligand-dependent "shedding" of N^{EC}. For instance, cell adhesion mediated by Notch/Ligand interactions has been shown to trigger an endocytic flow of Delta molecules in the Notch expressing cells, where it is eventually found in multivesicular bodies (Fehon et al., 1990, Cell 61:523-534; R. Fehon and S. Artavanis-Tsakonas,

unpublished results). Detailed expression studies of Delta expression in cells known to undergo Notch signaling are consistent with the cell culture findings (Kooh et al., 1993, Development 117:493-507). At this stage it seems that the simplest working hypothesis on Notch signaling should involve the heterodimeric (N^{EC}/N^{TM}) surface Notch complex proposed here, rather than the action of any single cleaved fragment (see the proposed model in Figure 9). The negative complementation displayed by the *Abruptex* mutation, a group of gain-of-function mutants affecting amino acids in the EGF homologous region of Notch, has been thought to reflect homotypic interactions between Notch receptors (Foster, 1975, Genetics 81:99-120; Xu et al., 1990, Genes Dev. 4:464-475). Therefore akin, for example, to the insulin receptor, the N^{EC}/N^{TM} heterodimer may be engaged in homotypic, or conceivably heterotypic, interactions. The analysis of the Notch receptor on nonreducing gels is consistent with this notion. In the absence of reducing agents, N^{EC} and N^{TM} are not detected. However, instead of detecting the full length molecule we detect higher molecular weight complexes of a yet undetermined nature (data not shown).

Since full-length Notch appears to reflect a ligand inaccessible, intracellular form of the protein, cleavage provides an important tool to regulate the Notch pathway. Such cleavage can effectively control the number of active surface receptors. Genetic analysis in *Drosophila* has demonstrated that the animal is unusually sensitive to the number of wild type copies of the Notch gene. In fact, Notch is one of a handful of genes in *Drosophila* that are both haplo insufficient as well as triplo mutant (Lindsley and Zimm, 1992, The genome of *Drosophila melanogaster*, (Academic Press, San Diego).

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the

art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.